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p53-dependent and p53-independent Activation of Apoptosis in Mammary Epithelial Cells Reveals a Survival Function of EGF and Insulin

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Abstract. The p53 tumor suppressor protein has been implicated as a mediator of programmed cell death (PCD). A series of nontransformed mammary epithelial cell (MEC) lines were used to correlate p53 function with activation of PCD. Treatment of MECs expressing mutant, inactive, or no p53 with DNA-damaging agents did not induce apoptosis. Upon introduction of temperature-sensitive p53 into HC11 cells, which lack wild-type (wt) p53, PCD was observed after mitomycin treatment at 32°, when the ts p53 protein is in wt conformation. Thus, wt p53 mediates activation of PCD in response to mitomycin in HC11 cells. Treatment of the MCF10-A cells, which express wt p53, with various DNA-damaging agents led to nuclear accumulation of p53. Only mitomycin treatment led to an increase in the number of apoptotic nuclei. ErbB-2–transformed MCF10-A cells responded to mitomycin, cisplatin, and 5-Fl-uracil, suggesting that signaling from activated ErbB-2 enhances the cells ability to respond to DNA damage. A combination of high cell density and serum-free medium induces apoptosis in all MECs tested, irrespective of their p53 status. Under these conditions, EGF or insulin act as survival factors in preventing PCD. These data might elucidate some aspects of breast involution and tumorigenesis.

THE p53 tumor suppressor protein is a potent inhibitor of cell growth and transformation, and is involved in the cellular response to DNA damage (reviewed in Zambetti and Levine, 1993; Lane, 1992). After irradiation- or chemically induced DNA damage, wild-type (wt)[1] p53 rapidly accumulates in the cell nucleus (Fritsche et al., 1993; Hall et al., 1993), and it orchestrates a number of nuclear events leading to a cell cycle arrest at the G1/S phase transition (Kastan et al., 1991; Kuerbitz et al., 1992). One of these events is the transcriptional activation of the p21/*waf*-1/*pic*-1 cyclin inhibitory gene (El-Deiry et al., 1993). During this cell cycle arrest, the cells would be allowed to repair the damaged DNA and then reenter the cell cycle (Lane, 1992). The DNA damage-induced cell cycle arrest requires wt p53 function (Kastan et al., 1991; Kuerbitz et al., 1992). A number of experiments have also indicated that wt p53 can mediate DNA damage-induced programmed cell death (PCD), presumably when the damage is excessive and incompatible with DNA replication (Barry et al., 1990; Kastan et al., 1991; Kuerbitz et al., 1992; Lowe et al., 1993*a*, *b*). Perhaps the most convincing evidence is that thymocytes from p53-deficient mice fail to undergo PCD after irradiation (Clarke et al., 1993; Lowe et al., 1993*b*).

The importance of understanding the involvement of the p53 tumor suppressor protein in the activation of PCD lies in the high frequency of p53 mutations found in human cancer (Hollstein et al., 1991), including breast cancer (Harris, 1992; Osborne et al., 1991). Potent DNA-damaging agents are commonly used in cancer chemotherapy (Calabresi and Chabner, 1993), and tumor regression after chemotherapy is caused, at least in part, by the ability of DNA-damaging drugs to activate PCD of the neoplastic cells. Absence of wt p53 might abolish this response to DNA damage and result in drug resistance. Most of the data in the literature were obtained from thymocytes (Clarke et al., 1993), lymphoid, or fibroblast cells (Lowe et al., 1993*a*, *b*) and very little is known for mammary epithelial cells (MEC). Since breast carcinoma represents the most frequent neoplasia in women, it is relevant to examine the mechanisms of cell death in mammary cells.

PCD is an active cellular process that is involved in a variety of physiological events in which rapid elimination of unwanted cells must occur. Its importance in the turnover of self-renewing tissues, morphogenesis, embryonic development, maturation of cells of the immune system (reviewed in: Kerr et al., 1972; Wyllie, 1992; Cohen et al., 1992; Raff,

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1. *Abbreviations used in this paper:* GM, growth medium; MEC, mammary epithelial cell; NAN, number of apoptotic nuclei; PCD, programmed cell death; PI, propidium iodide; SFM, serum-free medium, ts, temperature-sensitive; wt, wild-type.

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1993), as well as in the development of cancer and other pathologies (reviewed in: Sen and D'Incalci, 1992; Barr and Tomei, 1994), is increasingly being recognized. During the differentiation cycle of the mammary gland, massive apoptosis occurs at the cessation of lactation and leads to rapid involution of the gland to its puberal state (Walker et al., 1989; Strange et al., 1992). Features of cells undergoing PCD include chromatin condensation and nuclear fragmentation, internucleosomal DNA cleavage, shrinking, and appearance of apoptotic bodies, the hallmarks of a process also known as apoptosis (Kerr et al., 1972; Wyllie, 1980, 1984). Apoptosis is physiologically triggered by extracellular signals of a different nature, which appear to be cell type specific. The data indicate that both negative and positive apoptotic signals exist which can, respectively, prevent or induce PCD. Certain cell types are dependent on and compete for extracellular factors for their survival, which act by preventing the execution of PCD (Raff, 1993). Several examples have now been reported (Barres et al., 1992; Araki et al., 1990; Aisenman and deVellis, 1987; Drago et al., 1991; Geier et al., 1992; Kyprianou et al., 1991; Williams et al., 1990; Rodriguez-Tarduchy et al., 1990). Similarly, cells can be dependent on contact with extracellular matrix components for their survival (Meredith et al., 1993; Ruoslahti and Reed, 1994; Frisch and Francis, 1994).

In the present study, we report the existence of two pathways that lead to activation of PCD in MECs: one that is activated by DNA damage and requires the function of wt p53, and another that is induced by cell density and withdrawal of serum and that is independent of wt p53 activity. Starvation-induced apoptosis can be prevented by addition of insulin or EGF, indicating that these polypeptides are mammary cell survival factors. These observations could be used to construct models of regulation of PCD in MECs, characterize PCD signal transduction pathways, and address the issue of regulation of PCD in the mammary gland in vivo.

Materials and Methods

Cell Lines, Tissue Culture, and Transfection

The human breast epithelial cell lines MCF10-A (Soule et al., 1990) and MTSV1.7 (Bartek et al., 1991), as well as the rodent MEC lines HC11 (Ball et al., 1988) and Nulli B (courtesy of Dr. D. Medina, Baylor College of Medicine, Houston, TX), were used in this study. With the exception of the MTSV1.7 cells that were immortalized by expression of SV40-T antigen (Bartek et al., 1991), all these cell lines are spontaneously immortalized MEC lines derived from primary culture of mammary gland explants. To different extents, all these cells have retained some characteristics of normal MECs, and they are not transformed. The MCF10-A *erb*B-2 cells were derived from the MCF10-A cells by the introduction of an expression vector containing the human c-*erb*B-2 protooncogene (Ciardiello et al., 1992). The MCF10-A and MCF10-A *erb*B-2 cells were maintained in 1:1 DME/Ham's F12 growth medium (GM), supplemented with 10% fetal horse serum, EGF (10 ng/ml), insulin (5 μg/ml), hydrocortisone (10^{-6} M), cholera enterotoxin (100 ng/ml), glutamine, and gentamycin, as described (Soule et al., 1990). The MTSV1.7 cells were maintained in DME supplemented with 10% FBS, insulin (10 μg/ml), hydrocortisone (5 μg/ml), glutamine, and gentamycin, as described (Bartek et al., 1991). The HC11 and Nulli B cells were maintained in RPMI 1640 GM supplemented with 8% FBS, EGF (10 ng/ml), insulin (5 μg/ml), glutamine and gentamycin, as described (Ball et al., 1988). The HL60 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, glutamine, and gentamycin, and used as a positive control for apoptosis (Kaufmann, 1989). Serum-free medium (SFM) was prepared from DME/F12 (for MCF10-A and MTSV1.7 cells) or RPMI medium (for HC11 and Nulli B cells) by the addition of fetuin (0.5 mg/ml), transferrin (10 μg/ml), glutamine, and gentamycin.

The HC11 cells were cotransfected with the temperature-sensitive (ts) p53 expression vector LTRp53cGVal-135 (Michalovitz et al., 1990) and the pBabe-*puro* reporter plasmid, by the calcium phosphate precipitation method, as described in Chen and Okayama (1987) and Merlo et al. (1994). Transfected pools of cells were selected and maintained in RPMI medium containing 1 μg/ml of puromycin (Sigma Immunochemicals, St. Louis, MO). Integration of the ts-p53 vector was confirmed by Southern blot analysis, using a ^{32}P-labeled mouse p53 cDNA as a probe to hybridize on BamHI-digested DNAs, as described in Merlo et al. (1994).

Determination of Optimal Drug Concentration

The optimal concentrations of mitomycin C, cisplatin, 5-Fl-uracil, adriamycin, and vincristine were determined experimentally by measuring the total number of dead cells after treatment with increasing concentrations of the drugs. The Nile blue exclusion technique was used, as described (Jeffs and Osmond, 1992). Equal number of cells were plated on chamber slides (LabTek; Nunc Inc., Naperville, IL), incubated 24 h at 37°, and then treated with mitomycin C (Sigma) (1, 10, and 80 μg/ml), cisplatin (Platinol; Bristol-Myers Oncology Division, Evansville, IN) (1, 5, and 20 μg/ml), 5-Fl-uracil (Roche Laboratories, Nutley, NJ) (1, 10, and 50 μg/ml), adriamycin (Adriblastine; Farmitalia, Milan, Italy) (1, 10, and 50 μg/ml), and vincristine (Oncovin; Eli Lilly and Co., Indianapolis, IN) (1, 10, and 80 μg/ml) for 3, 6, or 12 h at 37°. Cells were then washed with P & C saline solution, stained with 1 mg/ml of Nile blue sulphate (Sigma) in P & C solution for 30 min at room temperature, washed twice in P & C solution for 1 h, and scored by microscopy. For all cell lines, after a 12-h treatment with the maximal drug concentration, <10% of the cells could be stained with Nile blue, indicating a level of cell viability >90% under these conditions. For detection of p53 protein by immunohistochemistry, the following concentrations were used: mitomycin C, 20 μg/ml; cisplatin, 5 μg/ml; 5-Fl-uracil, 10 μg/ml; adriamycin, 10 μg/ml; and vincristine, 10 μg/ml. For the induction of apoptosis, the following concentrations were used: mitomycin C, 40 μg/ml; cisplatin, 10 μg/ml; 5-Fl-uracil, 20 μg/ml; adriamycin, 20 μg/ml; vincristine, 20 μg/ml. Using these concentrations, >95% of the MCF10-A and MTSV1.7 cells and >98% of HC11 and Nulli B cells remained viable.

p53 Immunohistochemistry

Immunohistochemical detection of p53 protein was performed on cells plated on chamber slides (LabTek) and treated for 6 or 12 h with the DNA-damaging agents at the concentrations indicated above. The cells were washed with PBS three times, fixed with acetone/methanol 1:1 at −20°C for 10 min, washed three times with PBS, and stained for p53 using the p53-specific antibodies Ab2 (180l), Ab3 (240), and Ab6 (Do-1) (Oncogene Science Inc., Manhasset, NY). The Ab2 and Ab6 antibodies recognize an epitope common to wt and (most) mutant p53 proteins. The Ab3 (240) antibody recognizes an epitope common to (most) mutant but not to the wt p53 protein. For the murine cell lines HC11 and Nulli B, the rabbit polyclonal antibody CM-1 (Novocastra Laboratories, Newcastle, UK) was used. The slides were incubated 16 h with the antibody, at the dilution recommended by the manufacturer, then a biotinylated anti-mouse or anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) was applied and detected with the conventional avidin-biotin complex method, using diaminobenzidine as chromogen. The positive nuclei were scored by microscopy, and the results were expressed as percentage of positive nuclei over total number of nuclei counted. Untreated MCF10-A cells were also stained as negative controls.

Cell Treatment and Detection of Apoptotic Nuclei

Control experiments were initially carried out on the HL60 cells. These cells are known to respond to mitomycin C and undergo apoptosis with typical DNA laddering (Kaufmann, 1989). HL60 cells were treated with mitomycin C (10 μg/ml) for 30 min, fixed and stained with propidium iodide (PI) as described below, and examined by fluorescent miscroscopy. Nuclei with features characteristic of apoptosis were readily observed. These nuclei typically appeared shrunk, condensed, fragmented, and often "exploded" to generate characteristic apoptotic figures (Kerr et al., 1972; Wyllie, 1980, 1984). Apoptotic bodies were also observed.

The mammary cells were plated on polystyrene chamber slides (LabTek) 1 or 2 d before the treatment and incubated at 37°C. The medium was then replaced with freshly prepared drug-containing medium (mitomycin C, 40

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μg/ml; cisplatin, 10 μg/ml; 5-Fl-uracil, 20 μg/ml, adriamycin, 20 μg/ml; and vincristine, 20 μg/ml), and the incubation was continued for 3 h at 37°C. The cells were then fixed with cold 2% formaldehyde in PBS, for 10 min at 4°C, washed once with cold PBS, and the nuclei were stained with a solution containing 50 μg/ml PI, 0.1% Triton X-100, 0.1% Na citrate, and 20 μg/ml of RNase A in PBS for 15–20 min at room temperature. Apoptotic nuclei were counted within 2 h by examination of their morphology by a fluorescent microscopy using a Zeiss Axiophot microscope equipped with Neofluar 40× objective lens. The results were expressed as percentage of the number of apoptotic nuclei (NAN) over the total number of nuclei examined. A minimun of 1,000 nuclei were counted in each case.

Induction of Apoptosis by Density and SFM

Cells were trypsinized and plated on polystyrene chamber slides (LabTek) in the GM described above. When the cultures reached confluency, the medium was removed, cells were washed once with PBS and either SFM, SFM + EGF (10 ng/ml), SFM + insulin (5 μg/ml), SFM + EGF + insulin, or GM was added. The cells were then incubated for additional 48 h, fixed, stained with PI, and scored as described above.

Extracellular matrix-coated chamber slides were prepared using HC11 cells. The cells were grown to confluency in GM, then maintained in GM or switched to SFM for 48 h, and detached with 50 mM EDTA in PSB, essentially as described (Chammas et al., 1994).

Analysis of the DNA Fragmentation

Control experiments for the analysis of DNA fragmentation were initially carried out on the HL60 cells. 1 million HL60 cells were treated with mitomycin C for 30 min, collected by centrifugation at 4°C, washed with cold PBS, and used for DNA extraction, as described below. Upon treatment with mitomycin C, the characteristic 180-bp nucleosomal DNA laddering was readily observed.

HC11 cells were plated in 60-mm dishes (Costar Corp., Cambridge, MA), incubated in GM at 37°C, and then switched to SFM or to SFM + EGF for 48 h as described above. At the end of the incubation time, the cells were scraped from the dishes, combined with the supernatant, and collected by centrifugation at 4°C. The pellets were washed once with PBS and then either frozen at −70°C or immediately used for DNA extraction.

DNA was extracted as follows: 200 μl of lysis buffer containing 10 mM Tris-HCl, pH 7.8, 10 mM EDTA, 5 mM EGTA, 0.5% SDS, 0.1% β-mercaptoethanol, 50 μg/ml Proteinase K, and 50 μg/ml RNaseA were added and the solution was incubated at 37°C for 2 h. The DNA was purified by phenol/chloroform extraction, ethanol precipitated, washed, dried, dissolved in 20 μl of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), and loaded on 1.6% agarose gels. After electrophoresis, the DNA fragments were visualized by ethidium bromide staining, and the gels were photographed.

Results

p53 Status in Mammary Epithelial Cell Lines

The MCF10-A human breast cells express wt p53, as demonstrated by sequence analysis of the p53 cDNA and by single-strand conformation polymorphism analysis of the p53 gene (Diella et al., 1993). Staining with p53 antibodies is also consistent with the presence of wt p53 (see below). The MTSV1.7 human breast cells were established from primary cultures by the introduction of a vector expressing SV-40 T large antigen (Bartek et al., 1991), which is known to bind to and inactivate endogenous wt p53 (Ludlow, 1993). The murine HC11 cells contain two allelic mutations in their p53 gene and no wt p53 (Merlo et al., 1994). Accordingly, overexpression of p53 protein is detected with the CM-1 p53 antiserum (data not shown). The Nulli B cells were obtained from mammary gland explants of mice with a homozygous p53 gene knock-out (Donehower et al., 1992) and, therefore, are devoid of any endogenous p53 protein. Accordingly, no p53 protein was detected by immunohistochemistry (data not shown).

p53 is Required for DNA Damage-induced PCD in HC11 Cells

Wild-type p53 is thought to have a role in detecting DNA damage and in activating PCD. We have tested MECs with different p53 status for drug-induced PCD. Treatment of the HC11 cells plated at various densities with mitomycin C, cisplatin, 5-Fl-uracil, adriamycin, and vincristine at sublethal doses did not induce any increase in the NAN (data not shown). Thus, the absence of functional wt p53 in HC11 cells correlates with their inability to respond to DNA-damaging agents in the activation of PCD.

The HC11 cells were transfected with an expression vector containing ts p53 (Michalovitz et al., 1990; Merlo et al., 1994). The ts p53 vector expresses a p53 protein that remains mostly in the mutant conformation at 37°C and is mostly in the wt conformation at 32.5°C. After transfection and selection, DNA was extracted from pools of resistant cells and tested for the presence of ts p53 vector by Southern blot analysis. The expected hybridization fragments corresponding to ts p53 were observed in the transfected cells (Fig. 1 *B*). The endogenous p53 gene and pseudogene were also observed (Fig. 1 *B*).

The ts p53 HC11 cells were then maintained at 37°C or at 32°C for 48 h and tested for apoptosis. Parental HC11 cells were also maintained at 37°C and 32°C for comparison. While parental HC11 cells showed no difference in the NAN at 32°C or at 37°C, a 2.5-fold increase in the NAN was observed between ts p53-transfected HC11 cells maintained at 32°C vs 37°C (Fig. 1 *C*). These data indicate that wt p53 can by itself be a weak activator of PCD in these cells.

Parental and ts p53-transfected HC11 cells were then maintained at 37°C or at 32°C, and were tested for DNA damage-induced apoptosis using mitomycin C, cisplatin, 5-Fl-uracil, and adriamycin. At the same time, the cells were also tested with the non-DNA damaging agent vincristine. The parental HC11 cells maintained at 37°C or 32°C remained unresponsive to the drug treatment (Fig. 1 *C*). The ts p53 HC11 cells maintained at 37°C also did not respond to any treatment. In contrast, the ts p53-transfected cells maintained at 32°C showed a threefold increase in NAN over the untreated cells after treatment with mitomycin C (Fig. 1, *A* and *C*). This level of activation of PCD is comparable to that seen with the MCF10-A cells (see below). Only a slight increase in the NAN was seen with cisplatin and none with 5-Fl-uracil, adriamycin (data not shown), or vincristine. This result indicates that in HC11 cells the activation of apoptosis in response to mitomycin C–induced DNA damage requires a wt p53 function.

The Nulli B and the human cell line MTSV1.7, which also lack functional wt p53, were tested with the same DNA-damaging agents, and as seen for the HC11 cells did not show an increase in NAN (data not shown).

wt p53 Accumulates in Response to DNA Damage in MCF10-A Cells

The p53 specific antibodies Ab2 (Ab1801) and Ab6 (DO-1) were used to detect the p53 protein in MCF10-A cells by immunohistochemistry. These antibodies recognize wt and (most) mutant p53 proteins. Using the Ab2 and Ab6 antibodies, untreated MCF10-A cells showed very few p53-positive cells (<3%) (Fig. 2; Table I). These results are consistent

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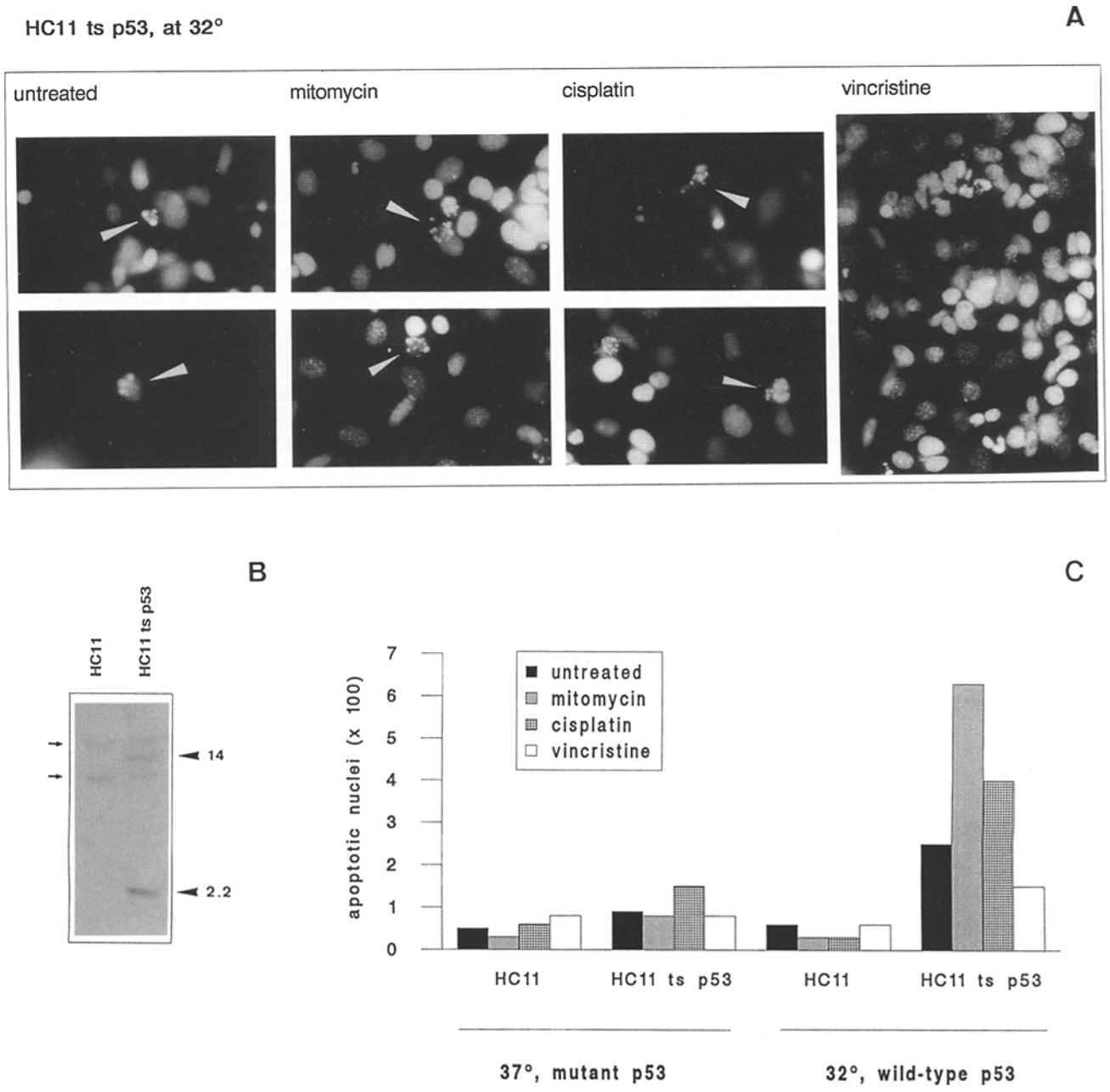

Figure 1. Apoptosis in HC11 cells transfected with the ts-p53 vector after treatment with DNA-damaging agents. (*A*) Photomicrography of PI-stained nuclei from untreated ts-p53 HC11 cells (*left*), compared to cells treated with (*left to right*) mitomycin C, cisplatin, and vincristine, at 32°C. At this temperature, the ts-p53 protein is mostly in wt conformation (Michalovitz et al., 1990). Nuclei with typical morphological features of apoptosis are indicated (*white arrows*). (*B*) Southern blot analysis of genomic DNA from parental (*left lane*) and ts-p53–transfected HC11 cells (*right lane*) hybridized with labeled mouse p53 cDNA. The endogenous p53 gene and pseudogene can be observed in both DNA samples (*thin arrows on the left*). The 14- and a 2.2-kb hybridizing fragments diagnostic of the ts-p53 vector (*thick arrows on the right*) are visible in the transfected cells. (*C*) NAN after treatement of parental and ts-p53 HC11 cells with mitomycin C (*hatched bars*), cisplatin (*gridded bars*), or vincristine (*open bars*) at 37°C (*left half*) or at 32°C (*right half*), compared to the untreated cells (*solid bars*). The NAN is expressed in percentages as the number of apoptotic nuclei over the total number of nuclei counted. Standard deviation is in all cases within ±10%.

with the presence of wt p53 in these cells. This low level of p53 positivity is probably caused by posttranslational stabilization of p53 protein that does not reflect the presence of a mutation, as described for other cell systems (Delmolino et al., 1993; Lehman et al., 1993; Vojtesek and Lane, 1993). Using the Ab3 (Ab240) antibody, which recognizes (most) mutant forms of p53 but not the wt p53, MCF10-A cells showed no staining, as expected (data not shown).

After treatment of the MCF10-A cells with the DNA-damaging agents mitomycin C, 5-Fl-uracil, and adriamycin, respectively, a DNA alkylating agent, a nucleoside analogue, and a topoisomerase II–dependent intercalating agent (Cala-

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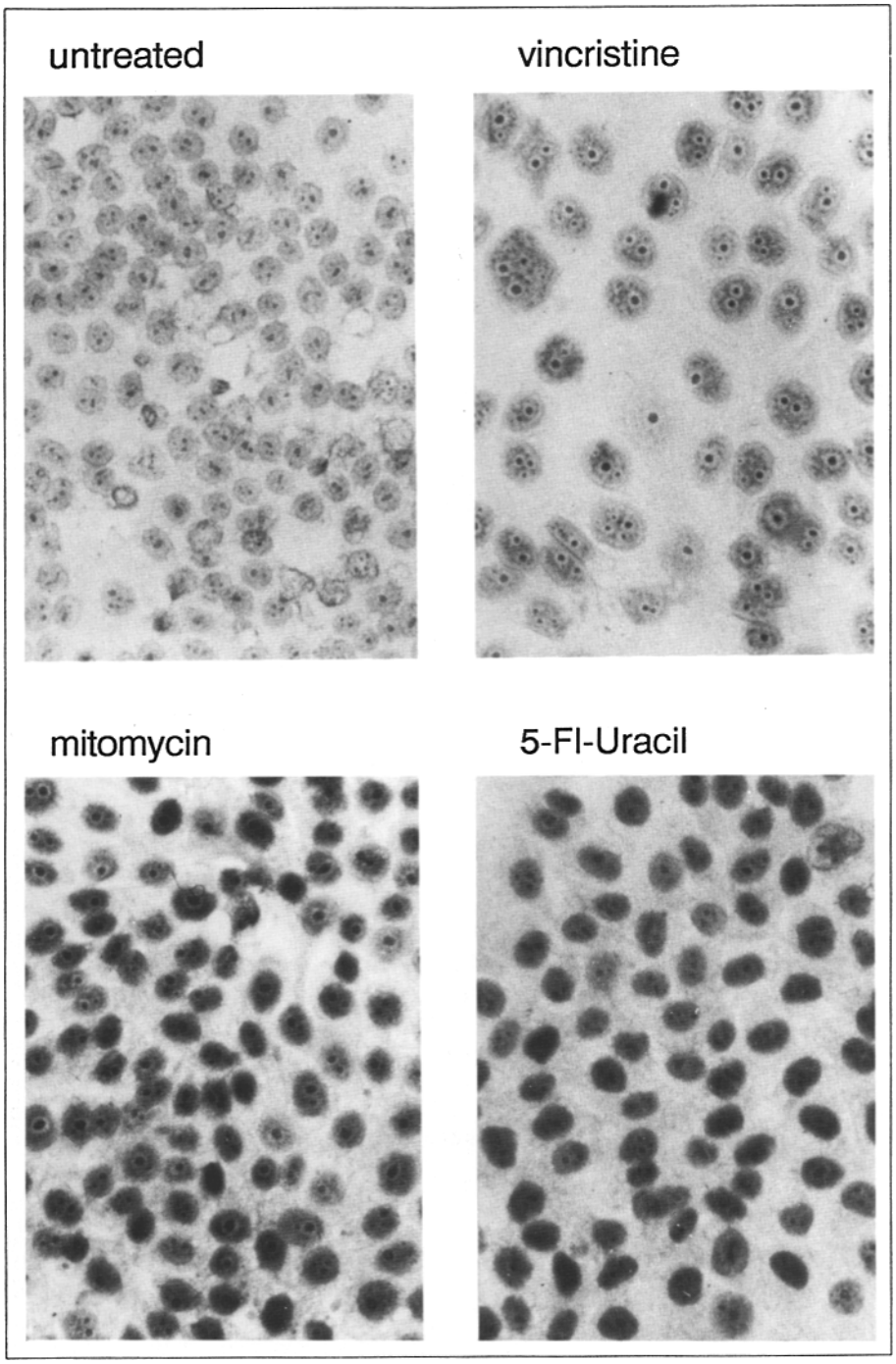

Figure 2. p53 immunoreactivity of MCF10-A cells treated with DNA-damaging agents. Results with the Ab2 anti-p53 antibody are shown. Treatments are for 6 h. Untreated cells (*top left*) and cells treated with vincristine (*top right*), a non-DNA-damaging drug, showed few p53 immunoreactive nuclei. Cells treated with mitomycin C and 5-Fl-uracil (*bottom, left* and *right*) showed intense p53 immunostaining.

bresi and Chabner, 1993), the number of p53-positive nuclei markedly increased (Fig 2; Table I). Using either antibodies Ab2 or Ab6, 80–90% of the nuclei were strongly positive for p53 after 12 h of treatment, a 26–30-fold increase over the untreated cells. After 6 h of treatment, a 24-, a 14-, and a 27-fold increase in the number of positive nuclei was observed, respectively, with mitomycin C, 5-Fl-uracil, and adriamycin. Cisplatin, an intercalating agent that causes breaks in the DNA (Calabresi and Chabner, 1993), was less efficient, and induced only a sevenfold increase in the number of p53-positive nuclei over untreated cells after 6 h of treatment (Table I). Technical reasons prevented long-term treatment of MCF10-A cells with cisplatin.

Treatment with the non–DNA damaging agents vincristine and methotrexate, respectively, an inhibitor of microtubules function and an inhibitor of purine biosynthesis (Calabresi and Chabner, 1993), for 6 or 12 h resulted in only a slight increase in the number of p53-positive nuclei (three- and eightfold higher than untreated cells after 6 and 12 h of treatment, respectively, Fig. 2; Table I). Thus, chemically induced DNA damage efficiently leads to the accumulation of immunoreactive p53 in the nucleus of MCF10-A cells.

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Table I. Expression of p53 in MCF10-A Cells after Treatment with DNA-damaging Agents

	p53 expression*	
	Treatment time	
Drug	6 h	12 h
Untreated	<3%	<3%
Mitomycin	71%	93%
Cisplatin	21%	ND‡
5-Fl-uracil	42%	83%
Adriamycin	82%	75%
Methotrexate	9%	25%
Vincristine	8%	25%

* The data shown here were obtained with the Ab2 antibody. The results with the Ab6 antibody were essentially indentical. Staining with the Ab3 antibody gave negative results in all cases.
‡ All cells detached from the slide after 12 h treatment with cisplatin. p53 expression could not be determined.

DNA-damaging Agents Induce Apoptosis in MCF10-A Cells

Subconfluent cultures of MCF10-A cells were treated with mitomycin C (40 μg/ml), cisplatin (10 μg/ml), 5-Fl-uracil (20 μg/ml), adriamycin (20 μg/ml), and vincristine (10 μg/ml) for 3 h, and the NAN were recorded by PI staining and microscopic examination. Mitomycin C induced a fourfold increase in the NAN over untreated cells (Fig. 3). The effect of mitomycin C on MCF10-A cells was dose dependent in the concentration range 20–100 μg/ml (data not shown). The experiment was repeated on cultures of MCF10-A cells at high density with similar results (data not shown). Under the same conditions, cisplatin, 5-Fl-uracil, and adriamycin did not elicit an apoptotic response. This result was surprising since 5-Fl-uracil and adriamycin could efficiently induce accumulation of p53 in the MCF10-A nuclei. To rule out the possibility that this result was caused by a nonoptimal treatment regimen, the experiment was repeated with increasing drug concentration (20–120 μg/ml) and for longer time (3–12 h). None of these treatments led to an increase in the basal level of apoptosis seen in the untreated cells. At higher doses, instead, the drugs caused 100% necrotic cell death.

MCF10-A Cells Overexpressing c-erbB-2 Respond Differently to DNA Damage

Human breast cancer cells may differ in their apoptotic response to DNA-damaging agents from the normal epithelial cells. Since most cancer cell lines available contain mutations in their p53 gene, we chose to analyze in vitro–transformed MCF10-A cells, which express wt p53, for activation of PCD upon chemically induced DNA damage. The MCF10-A ErbB-2 cells were derived from the MCF10-A cells by the overexpression of the c-*erb*B-2 proto-oncogene, and they have acquired the ability to form colonies in soft agar (Ciardiello et al., 1992; Bianco et al., 1994).

The MCF10-A ErbB-2 cells were treated with mitomycin C, cisplatin, 5-Fl-uracil, adriamycin, and vincristine in the same conditions as for the MCF10-A cells, and the NAN was scored. Mitomicin C could elicit an apoptotic response comparable (sixfold over the untreated cells) to that seen with the MCF10-A cells (Figs. 3 *B* and 4). In addition, cisplatin and 5-Fl-uracil treatment of these cells led to, respectively, a 6.5- and a 3-fold increase in the NAN (Fig. 4), an effect not seen with the MCF10-A cells (Fig. 3 *B*). These data indicate that there might be a synergy between ErbB-2 signaling and DNA damage-induced p53 activation in PCD.

Induction of Apoptosis by Confluency and SFM

Cultures of MCF10-A, Nulli B, HC11 and MTSV1.7 MEC were maintained in GM, which contains FBS, EGF and insulin, allowed to grow to different levels of confluency, and switched to SFM. After 48 h in SFM, the cells were stained with PI and scored for the NAN. Little or no difference was observed in the NAN in subconfluent MCF10-A, Nulli B, and HC11 cells when these cells were switched to SFM (Fig. 5). With the exception of the MTSV1.7 cells, MECs grown to confluency in GM did not significantly undergo apoptosis over basal levels. In contrast, a remarkable increase in NAN was observed with all the MEC lines tested when the cells were allowed to reach confluency and then switched to SFM for 48 h (Fig. 5). The confluent MCF10-A, HC11, Nulli B, and MTSV1.7 cells in SFM showed a 8-, 7.5-, 8- and 3-fold increase in NAN, respectively, compared to confluent cells in GM. Since these various cell lines have a different p53 status, we conclude that SFM and confluency-induced apoptosis occurs independently of the function of wt p53.

We have previously shown that HC11 cells deposit ECM proteins, which contribute to their ability to differentiate (Chammas et al., 1994). Therefore, we examined whether cell–matrix interactions at confluency could induce the apoptotic signal. HC11 cells were grown to confluency and maintained either in GM or in SFM for 48 h. The cells were then removed from the culture dishes using a protocol that preserves the biological activity of extracellular matrix components (Chammas et al., 1994). Low numbers of HC11, MCF10-A, MTSV1.7, and Nulli B cells were then plated on the extracellular matrix-coated dishes and maintained either in GM or in SFM for 48 h. In no case did the exogenous extracellular matrix activate PCD in any of the sparse MEC lines (data not shown). This result makes it unlikely that changes in extracellular matrix composition or assembly in confluent cultures induce apoptosis.

EGF and Insulin Are Survival Factors

We then tested the ability of EGF and insulin to restore survival of MECs. As illustrated in Fig. 6, both EGF (10 ng/ml) and insulin (5 μg/ml) efficiently blocked starvation-induced PCD of the MCF10-A, the HC11, and the Nulli B cells, reducing the NAN to levels comparable to those observed when cells are maintained in GM. For these cell lines EGF seemed to be more potent than insulin. The MTSV1.7 cells were only partially protected by EGF. In all cases, the addition of EGF and insulin together completely protected the cells from apoptosis. Finally, DNA fragmentation analysis was carried out on DNA extracted from HC11 cells maintained in GM, SFM, or SFM supplemented with EGF (10 ng/ml). A characteristic ladder of nucleosomal-sized DNA fragments was visible in the DNA from cells maintained in SFM, while no such DNA ladder was observed when cells were maintained in GM or in EGF-supplemented SFM (Fig. 7). This result confirms the apoptotic nature of the effects described above.

No DNA laddering could be observed in the DNA ex-

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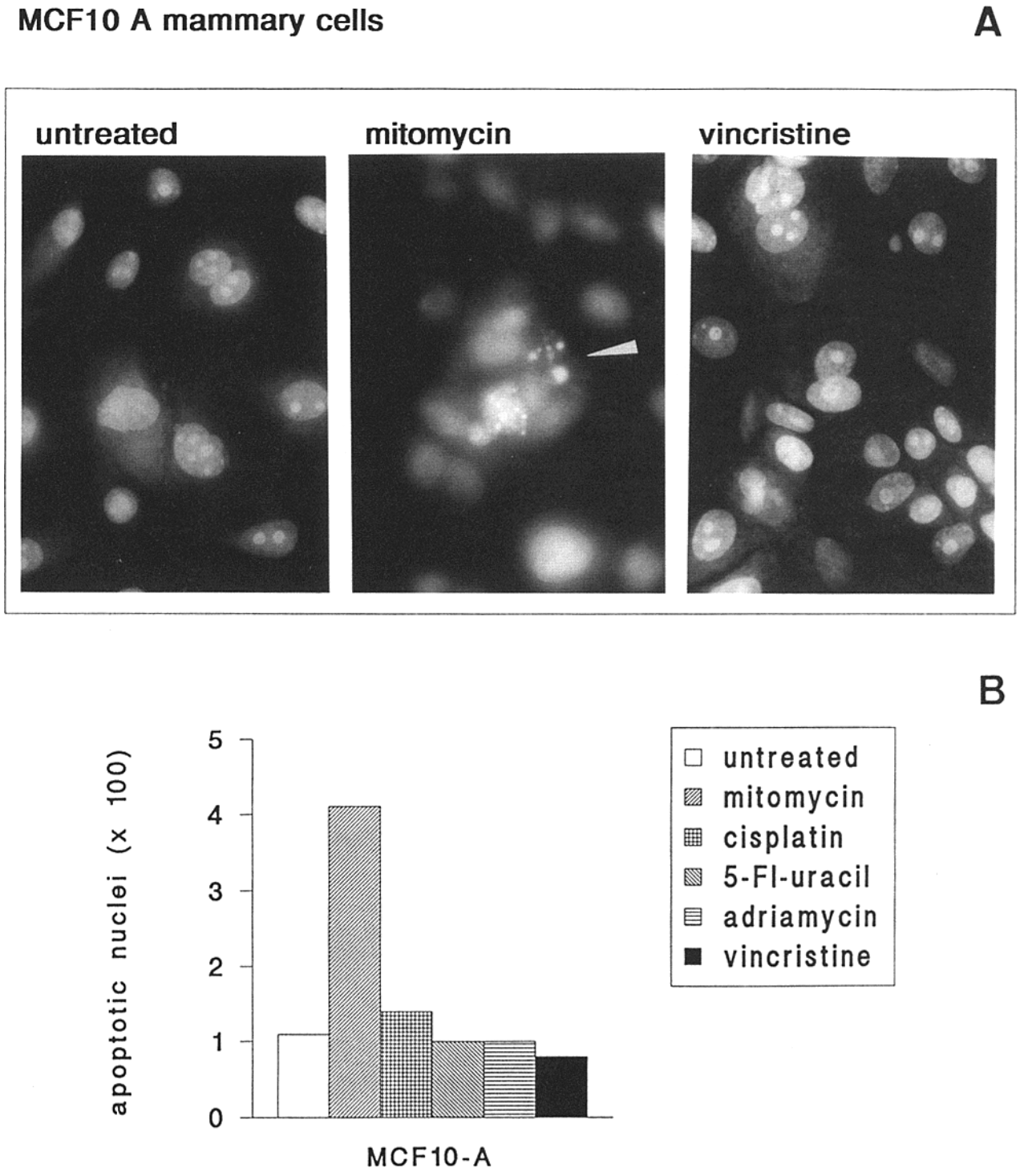

Figure 3. Detection of apoptosis in MCF10-A cells after treatment with DNA damaging agents. (*A*) PI staining of nuclei from untreated MCF10-A cells (*left*) compared to nuclei from cells treated with mitomycin C (*center*) and vincristine (*right*). An example of a nucleus with characteristic features of apoptosis is indicated (*white arrow*). (*B*) NAN in cultures of untreated MCF10-A cells (*open bar*) or cells treated with DNA-damaging agents. Legend to the drug treatments is shown in the panel. Treatment with mitomycin C induced a fourfold increase in NAN. The NAN is expressed in percentages as the number of apoptotic nuclei over the number of nuclei counted. □, untreated; ▨, mitomycin; ▦, cisplatin; ▧,5-Fl-uracil; ▤, adriamycin; ■, vincristine.

tracted from confluent Nulli B or MTSV1.7 cells maintained in SFM (data not shown). The reason for this is unknown, but it reinforces the idea that apoptosis can occur in the absence of detectable DNA laddering (Collins et al., 1992).

Discussion

The results presented indicate that two distinct pathways which differ in their requirement for wt p53 can lead to the

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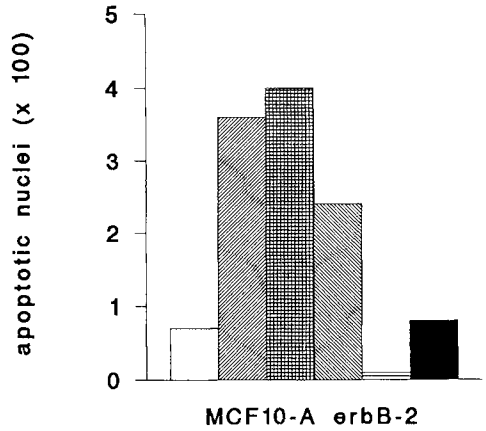

Figure 4. Detection of apoptosis in MCF10-A ErbB-2 cells after treatment with DNA-damaging agents. Legend to the drug treatments is the same as in Fig. 3 and is indicated in the panel. Treatment with mitomycin C, cisplatin, and 5-Fl-uracile induced, respectively, a 6-, 6.5-, and 3-fold increase in NAN. The NAN is expressed in percentages as the number of apoptotic nuclei over the number of nuclei counted. Symbols have the same representations as in Fig. 3.

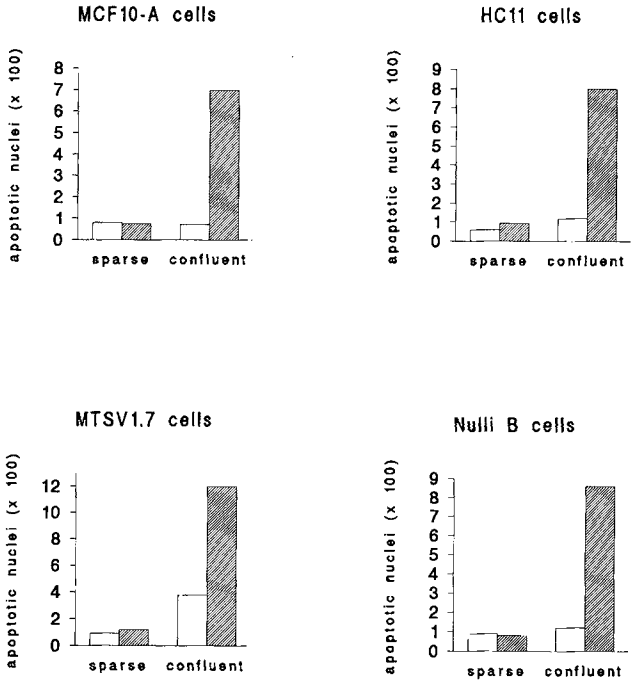

Figure 5. Apoptosis induced by cell density and SFM. Results with MCF10-A, HC11, MTSV1.7, and Nulli B cells are shown. Cells were plated in GM, allowed to reach different densities (sparse or confluent), then either switched to SFM (*hatched bars*) or maintained in GM (*open bars*) for an additional 48 h. The results obtained with sparse or confluent cultures are shown. The NAN is expressed in percentages as the number of apoptotic nuclei over the number of nuclei counted.

activation of PCD in nontransformed MECs. Thus, wt p53 is not required for all signaling pathways leading to apoptosis.

A role for wt p53 in detecting DNA damage is being increasingly recognized (Lane, 1992). Upon UV irradiation (Hall et al., 1993), γ-irradiation (Kastan et al., 1991; Kuerbitz et al., 1992; Lowe et al., 1993*b*), or treatment with DNA-damaging anticancer chemotherapy drugs (Fritsche et al., 1993; Lowe et al., 1993*a*), cellular p53 was shown to rapidly accumulate in the nucleus and to induce a cell cycle arrest. This would then allow for DNA repair to occur. Our results on MECs confirm this notion, since treatment of the MCF10-A cells with the DNA-damaging agents mitomycin C, 5-Fl-uracil, adriamycin, and to a lower extent, cisplatin, led to rapid nuclear accumulation of the p53 protein. At the same time, excessive DNA damage is known to commit the cell to PCD in a wt p53-dependent manner (Lane, 1992; Clarke et al., 1993; Lowe et al., 1993*a*). The results we have obtained on our panel of MEC lines confirm in part this function of wt p53, in that mitomycin C treatment led to a p53-dependent increase in apoptosis. It is intriguing, though, that treatment with 5-Fl-uracil and adriamycin did not elicit apoptosis in the MCF10-A cells, in spite of the rapid accumulation of wt p53 in the treated cells. High doses of 5-Fl-uracil and adriamycin caused necrotic cell death. Since these drugs induce DNA damage by different mechanisms (Calabresi and Chabner, 1992), the failure of these drugs to induce apoptosis may be caused by differences in the type and amount of DNA damage.

Interestingly, the MCF10-A cells transformed by high expression levels of ErbB-2 behaved differently from the parental MCF10-A cells. These cells responded to mitomycin C, cisplatin, and to a lesser extent, 5-Fl-uracil treatment with increased PCD. Thus, the PCD-stimulating activity of DNA-damaging drugs does not only depend on wt p53, but can be modulated by activating the ErbB-2 receptor tyrosine kinase. In fact, ErbB-2–specific agonistic antibodies have been shown to increase the cytotoxicity of cisplatin treatment on human breast carcinoma cells. Receptor signaling appears to be required for this effect (Arteaga et al., 1994). This observation is of great interest in oncology since c-*erb*B-2 is amplified and/or overexpressed in a significant subset of human breast cancers. Tumors that harbor c-*erb*B-2 amplification may have a defined sensitivity to chemotherapy involving the use of DNA-damaging drugs. If this is true, the regulation of PCD in cancer cells will become an area of intense research in oncology.

The requirement for wt p53 function in DNA damage-induced PCD in MECs was demonstrated by the lack of response in cells with altered p53 and the subsequent experiments in which introduction of wt p53 in HC11 cells restored their responsiveness to mitomycin C. These observations support a central role of wt p53 in activating PCD in response to DNA damage, although it is not a universal feature of mammalian cells. In fact, HL60 cells lack endogenous p53 (Kastan et al., 1991), and yet they rapidly undergo apoptosis after DNA damage (Kaufmann, 1989).

It has previously been reported that in myeloid and leukemia cell lines the introduction of wt p53 could induce high levels of apoptosis and a significant loss of cell viability (Shaw et al., 1992; Yonish-Rouach et al., 1991; Ryan et al., 1993). The expression of wt p53 in HC11 cells by itself could induce low levels of apoptosis, although the effect was more limited. This observation could reflect differences in cell type or could be explained by differences in the extent of DNA damage that the cells have accumulated in the absence of wt p53. The activity of exogenous p53 protein indeed de-

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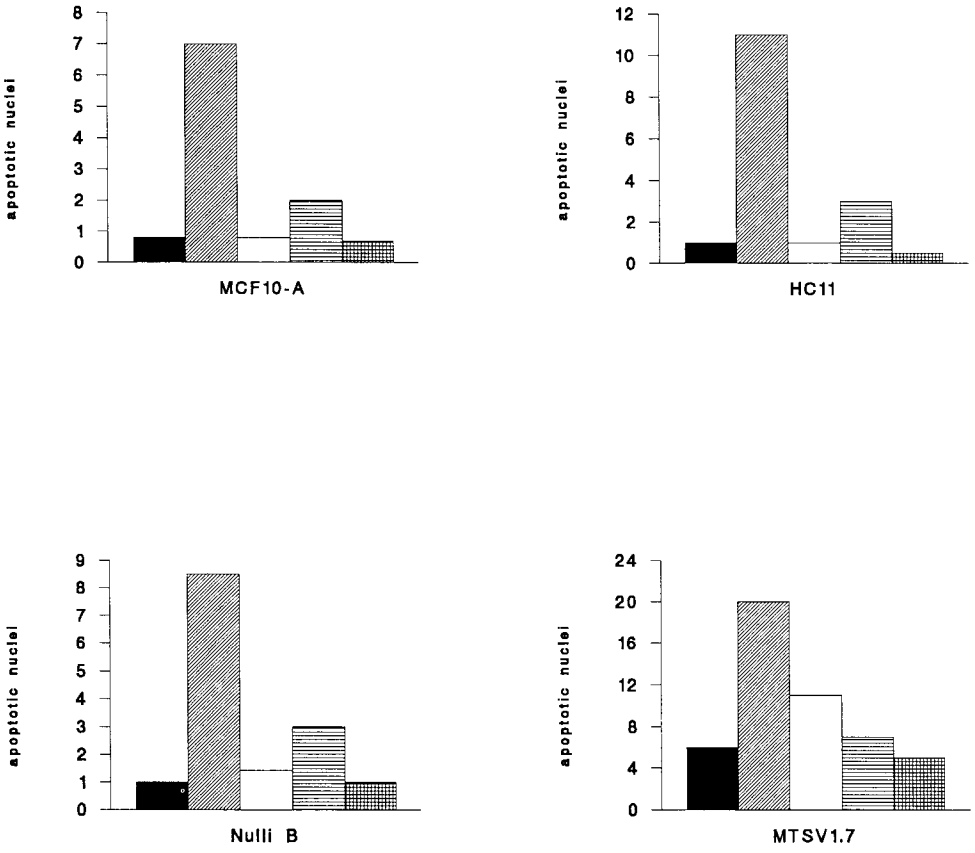

Figure 6. EGF and insulin as survival factors for mammary epithelial cells. The results obtained with the MCF10-A, HC11, MTSV1.7, and Nulli B cell lines are shown. Cells were plated and maintained in GM (*solid bars*) or switched to SFM (*hatched bars*), SFM + EGF (*open bars*), SFM + insulin (*horizontal bars*), or SFM + EGF + insulin (*gridded bars*) for 48 h. Both EGF and insulin at the concentration used (10 ng/ml and 5 μg/ml, respectively) showed a survival factor activity. The NAN is expressed in percentages as the number of apoptotic nuclei over the number of nuclei counted.

pends on the "cellular environment" in which it is expressed (Vojtesek and Lane, 1993).

The second pathway that activates PCD involves the combined effect of high cell density and SFM, and it is clearly independent of the wt p53 function. The existence of p53-independent pathways has also been shown in thymocytes (Clarke et al., 1993). In contrast, IL-3–dependent hematopoietic cells require p53 for both DNA damage- and starvation-induced apoptosis (Gottlieb et al., 1994). Starvation-induced PCD likely reflects the depletion of a cell-type specific survival factor(s). Examples of survival factors include PDGF for glial cells (Barres et al., 1992), FGF for endothelial cells (Araki et al., 1990) and for fibroblasts (Tamm et al., 1991), insulin-like growth factors (IFGs) for neuronal cells (Aisenman and deVellis, 1987; Drago et al., 1991; Barres et al., 1992) and breast cancer cells (Geier et al., 1992), estrogens for MCF-7 breast cancer cells (Kyprianou et al., 1991), and IL-3 for myeloid cells (Williams et al., 1990; Rodriguez-Tarduchy et al., 1990). The hypothesis that the GM contains MEC-specific survival factors was fully confirmed by subsequent experiments in which EGF and insulin were added to the SFM and shown to have this property. These properties are not unique to a particular cell line, but they appear to be a general property of MECs.

An interesting aspect of starvation-induced apoptosis is the fact that serum, insulin, and EGF deprivation are not sufficient by themselves to efficiently induce apoptosis. Subconfluent cultures of cells maintained in SFM responded very little or not at all, whereas starvation of the confluent cultures led to a large increase in PCD. Identical results, i.e., little or no activation of PCD, were obtained with subconfluent cultures of cells plated on an extracellular matrix deposited by HC11 cells induced to undergo PCD. These results argue for a primary role of cell–cell contact in the initial signaling for apoptosis. What the signal might be is cur-

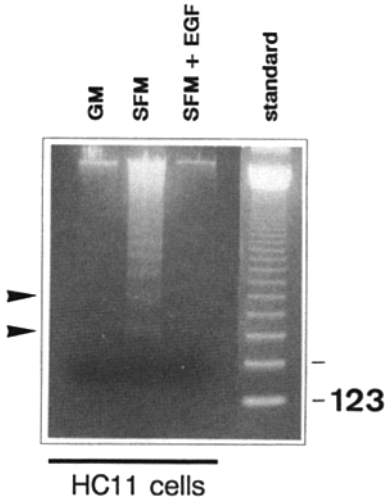

Figure 7. DNA fragmentation in the HC11 cells. Agarose gel electrophoresis of DNA from HC11 cells maintained in (*left to right*) GM, SFM, or SFM + EGF (10 ng/ml) for 48 h. A laddering of DNA fragments 180 bp or multiple in size, characteristic of apoptosis, was detected in the DNA from cells in SFM (*left arrows*), but not in the DNA from cells in GM. Addition of EGF to the SFM completely prevented this DNA fragmentation. Molecular weight marker (123-bp ladder) is shown on the right.

rently unknown, but cadherin-type molecules may be involved, and it is possible that cell density leads to changes in the status and function of surface receptors, as well as increased sensitivity to EGF and insulin depletion. A different role of cell–matrix interaction can be envisioned. In confluent cultures of mammary cells, integrin-like surface receptors may progressively lose the ability to contact their ligand, or the accessibility of extracellular matrix components might change (Ruoslahti and Reed, 1994). Cell contact signals might cause or accompany changes in the deposition or interaction with extracellular matrix components, which in turn sensitize the cells to EGF and insulin depletion. In kidney cells, for example, density and cell contact is necessary for efficient induction of apoptosis by disruption of cell–matrix interaction (Frisch and Francis, 1994). This and other reports support the idea that proper cell–matrix interaction can indeed be regarded as a survival factor (Meredith et al., 1993). The importance of cell–cell and cell–matrix contact in PCD of MEC will be addressed in the future.

During postlactational involution of the mammary gland, a large fraction of epithelial cells are eliminated by PCD (Walker et al., 1989; Strange et al., 1992), and this is accompanied by extensive tissue remodeling (Strange et al., 1992). Comparing the data presented here, obtained from cultured MECs with the physiological activation of PCD that occurs in vivo, the following similarity can be noticed: in vitro, serum depletion combined with cell density creates a condition of dependency on EGF or insulin for cell survival. In vivo, at the time of initiation of involution, the epithelial cells in the mammary gland are in tight contact with each other and almost completely occupy the parenchymal space (Walker et al., 1989; Strange et al., 1992). Whether a drop in the local levels of EGF (or other members of the EGF family of growth factors) and insulin (or IGFs) occurs and plays a role in the involution of the mammary gland remains to be established. Little is known about the sequence of signals that lead to PCD during mammary gland involution. However, intracellular signal transducers including PKA and AP-1 (*fos*/*jun*D) have been recently shown to be activated during involution (Marti et al., 1994). The results described here may provide a model system for PCD in MEC to help in the description of the molecular events involved in the breast involution process.

The data presented on SFM-induced activation of PCD clearly indicate that this effect is independent of wt p53 in vitro. It would be interesting to confirm this relationship in vivo, using homozygous p53 knock-out mice (Donehower et al., 1992) and examining whether their mammary glands are able to undergo normal differentiation and involution.

Compared to their normal counterparts, cancer cells may acquire neoplastic properties by losing their ability to respond to the apoptotic signals and consequently acquire novel survival capacity. Thus tumorigenesis may reflect the combined effect of growth promoting and antiapoptotic molecules (Mikulski, 1994). The neoplastic phenotype of breast cancer cells may be caused in part by the autocrine expression of antiapoptotic factors that prevent the tumor cells from entering PCD and provide indefinite survival capacity. The identification of insulin and EGF as MEC survival factors raises the possibility that EGF-like and insulin-like growth factors also play a role in human breast tumorigenesis. It has been shown that IGFs can prevent or delay apoptosis in several cell types (Barres et al., 1992; Aisenman and deVellis, 1987; Drago et al., 1991), including breast cancer cells (Geier et al., 1992). It has also been shown that IGF-II cooperates with SV-40 T large antigen during tumor progression by providing for an antiapoptotic signal rather than a mitogenic signal (Christofori et al., 1994). Breast cancer cells are known to express IGF-1 receptors on their surface (Cullen et al., 1990; Pekonen et al., 1988; Foekens et al., 1989), and high IGF I and II levels are found in breast cancer specimens, acting in a paracrine fashion (Yee et al., 1988, 1989; Cullen et al., 1991; Osborne et al., 1989). Similarly, *ras*-transformed MECs synthesize and secrete active TGF-α (Ciardiello et al., 1988), a peptide growth factor related to EGF, and human breast carcinomas express at least three EGF-related polypeptides, TGF-α, amphiregulin, and cripto-1 (Ciardiello et al., 1989; Qi et al., 1994), providing strong support for a role of EGF and EGF-related growth factors in breast carcinogenesis. Our data are consistent with the attractive possibility that breast cancer cells use IGFs and TGF-α to create an autocrine activation of antiapoptotic pathways. Whether oncogene-mediated in vitro transformation of the HC11 cells can modify their requirement for EGF or insulin for survival remains an open question and will be addressed.

Knowledge about the mechanisms of DNA damage–induced PCD also has implications in cancer management. In our in vitro system the alkylating agent mitomycin C can activate PCD in a p53-dependent manner. Loss, mutation, or inactivation of p53 in breast tumors may be correlated with resistance to a given chemotherapy that involves DNA damage. p53 mutations are found in a high fraction of ($\sim$30%), but not all human breast carcinomas (Harris, 1992; Osborne et al., 1991). There is hope that information about the status of p53 in a given tumor specimen might help predict patients' responses to a given DNA-damaging drug used in chemotherapy.

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